MOLECULAR ENGINEERING FOR CATALYTIC EFFICIENCY OF XYLANASE FROM *Aspergillus fumigatus* RT-1 AND ITS APPLICATION IN HYDROLYSIS OF PRETREATED KENAF

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy (Bioprocess Engineering)

School of Chemical and Energy Engineering
Faculty of Engineering
Universiti Teknologi Malaysia

MARCH 2020

ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my supervisor Prof. Dr. Rosli B. Md. Illias for the continuous support of my Ph.D. study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better mentor for my Ph.D. study.

My sincere thanks also go to Ministry of Science, Technology and Innovation (MOSTI) for providing a scholarship of MyBrain which support my fee expenses and allowances throughout five years of my study.

Besides, I would like to thank the rest of the committees from Malaysian Genome Institute which had provided courses and guides for technical knowledge regarding my studies.

I thank my fellow labmates in Genetic Lab from 2011 to 2019: Rabiatul Adawiyah, Izawati, Rohaida, Shalyda, Abbas, Izyan, Hasma, Baidura, Ling How Lie, Faizah, Samson, Hakimi, Yeng, Amalina, Joyce, Joanne, Aishah, Kheng Onn, Aizi Mazila, Anuar, Siti Fatimah, Namirah, Noradilin, Hidayah, Hamiza, Nardiah and Nashriq, for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last eight years.

Last but not the least, I would like to thank my family: my parents Rusnah Ibrahim and Damis Madek; my siblings: Nina, Mamat, Diana, Huda, Dinah, and Syifaa; and my dearest: Fairos, Khaleef, and Najla for endless loves and cares and supporting me spiritually and financially throughout my life.

ABSTRACT

The lignocellulose of industrial crops consists of three main polymers: cellulose, hemicellulose, and lignin. The combination of these complex and heterogeneous polymers contributes to the recalcitrant structure of lignocellulose. Thus, it becomes a drawback for a group of hydrolytic enzymes which work synergistically to hydrolyse the lignocellulosic substrate including xylanase. Hence, this study aimed to improve the catalytic efficiency of *Aspergillus fumigatus* RT-1 xylanase (AfxynG1) on pretreated kenaf hydrolysis through protein engineering of amino acids that located near the substrate-binding site and at the N-terminal region. Molecular docking analysis revealed 5 subsites; -3, -2, -1, +1, and +2 and several of substrate-binding residues which distributed alongside the subsites. Two putative binding residues of Phe 146 and Phe 30 and a putative secondary binding site of residue Tyr 7 were determined. High-throughput and low-throughput screenings of 5000 clones from error-prone PCR library which acted as fine tuner and 414 clones from site-saturation mutagenesis library were successfully performed to screen out three improved mutants; c168t, Q192H, and Y7L. The site-directed mutagenesis was applied to construct double and triple mutants and this process resulted in only two improved mutants; c168t/Q192H and c168t/Q192H/Y7L. The triple mutant c168t/Q192H/Y7L was the most stable enzyme in high temperature 60 and 70 °C and acidic pH 3-6, while the double mutant c168t/Q192H showed to contribute to the most effective hydrolysis reaction with a 7.6-fold increase in catalytic efficiency. Mutant Y7L produced the highest sugar yield with 28 % increase in pretreated kenaf hydrolysis. Overall, these improved mutants are feasible to be used synergistically with cellulases for bioconversion of lignocellulose into reducing sugar.

ABSTRAK

Lignoselulosa yang diperolehi daripada tanaman industri terdiri daripada tiga jenis polimer utama: selulosa, hemiselulosa dan lignin. Gabungan polimer-polimer yang kompleks dan heterogen ini menyumbang kepada struktur rekalsitran lignoselulosa. Maka, ia menjadi masalah bagi sekumpulan enzim hidrolitik yang bertindak secara sinergistik untuk menghidrolisis substrat lignoselulosa termasuk xilanase. Oleh itu, kajian ini dijalankan adalah bertujuan untuk meningkatkan kecekapan pemangkinan xilanase daripada *Aspergillus fumigatus* RT-1 (AfxynG1) ke atas hidrolisis kenaf terawat melalui kejuruteraan protein terhadap asid amino yang terletak berhampiran tapak pengikat substrat dan di kawasan N-terminal. Analisis dok molekul mendedahkan 5 tapak pengikat substrat; -3, -2, -1, +1, dan +2 dan beberapa residu pengikat substrat yang teragih sepanjang tapak pengikat substrat. Dua pengikat residu putatif iaitu Phe 146 dan Phe 30 serta satu residu tapak pengikat sekunder putatif Tyr 7 telah dikenalpasti. Penyaringan pemprosesan tinggi dan rendah terhadap 5000 klon daripada pustaka cenderung ralat PCR yang berperanan sebagai penala halus dan 414 klon daripada pustaka mutagenesis tapak penepuan berjaya menyaring tiga mutan yang ditambahbaik; c168t, Q192H, dan Y7L. Mutagenesis tapak terarah telah digunakan untuk membina mutan ganda dua dan ganda tiga yang mana proses ini menghasilkan hanya dua mutan ditambahbaik; c168t/Q192H dan c168t/Q192H/Y7L. Mutan ganda tiga c168t/Q192H/Y7L ialah enzim yang paling stabil pada keadaan suhu tinggi 60 dan 70 °C dan pH berasid 3-6 manakala mutan ganda dua c168t/Q192H telah menyumbang kepada reaksi hidrolisis paling efektif dengan 7.6 kali ganda peningkatan dalam kecekapan pemangkinan. Mutan Y7L menghasilkan gula tertinggi iaitu sebanyak 28 % peningkatan dalam hidrolisis kenaf terawat. Secara keseluruhannya, semua mutan yang ditambahbaik ini boleh digunakan secara sinergi dengan selulase untuk biopenukaran lignoselulosa kepada gula penurun.

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LIST OF ABBREVIATIONS

GH	-	Glycoside hydrolase
N-ter	-	N-terminus
C-ter	-	C-terminus
PCR	-	polymerase chain reaction
epPCR	-	error-prone PCR
GHG	-	greenhouse gas
AFLP	-	amplified fragment length polymorphism
MARDI	-	Malaysia Agricultural Research and Development Institute
EC	-	Enzyme Commission
CBM	-	carbohydrate-binding modules
SBS	-	secondary binding site
Asn	-	asparagine
Thr	-	threonine
Ser	-	serine
Trp	-	tryptophan
Arg	-	arginine
CMC	-	carboxymethyl cellulose
StEP	-	staggered extension process
mM	-	millimolar
dNTP	-	deoxynucleoside triphosphate
Mg^{2+}	-	ion magnesium
Mn^{2+}	-	ion mangan
DNA	-	deoxyribonucleic acid
Kb	-	kilobase

SSM	-	site-saturation mutagenesis
DMSO	-	dimethyl sulfoxide
DNS	-	3,5-dinitrosalicylic acid
PPA	-	polyphosphoric acid
ml	-	mililiter
h	-	hour
HTS	-	high-throughput screening
LTS	-	low-throughput screening
LB	-	Luria-Bertani
IPTG	-	Isopropyl β- d-1-thiogalactopyranoside
rpm	-	revolutions per minute
µl	-	microliter
min	-	minutes
nm	-	nanometer
OD	-	optical density
mg/ml	-	milligram per mililiter
g	-	gram
mg	-	milligram
BLAST	-	Basic Local Alignment Search Tool
RMSD	-	root mean square deviation
PDB	-	Protein Data Bank
3D	-	three-dimensional
PIC	-	Protein Interaction Calculator
ng	-	nanogram
U	-	enzyme unit
sec	-	seconds
µg	-	microgram
TAE	-	tris-acetate-EDTA
TSS	-	transformation and storage solution
$NiSO_4$	-	nickel sulphate
SDS-PAGE	-	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
V	-	voltan
w/v	-	weight per volume
RT	-	room temperature

BSA	-	bovine serum albumin
UV	-	ultra-violet
K_m	-	Michaelis-Menten constant
k_{cat}	-	turnover number
k_{cat}/K_m	-	specificity constant
OFAT	-	one factor at a time
mg/g	-	milligram per gram
Ala	-	alanine
GRAVY	-	grand average of hydropathicity
Lys	-	lysine
MW	-	molecular weight
DOPE	-	discrete optimized protein energy
Leu	-	leucine
Gln	-	glutamine
Tyr	-	tyrosine
Glu	-	glutamate
Sec.	-	section
X2	-	xylobiose
X3	-	xylotriose
X4	-	xylotetraose
X5	-	xylopentaose
X6	-	xylohexaose
kcal/mol	-	kilo calorie per mole
Phe	-	phenylalanine
Val	-	valine
Pro	-	proline
Ile	-	isoleucine
Asp	-	aspartate
His	-	histidine
Gly	-	glycine
SDM	-	site-directed mutagenesis
wild-type	-	wild-type
Ni-NTA	-	nickel-NTA
Cys	-	cysteine

Rsmax	-	maximum reducing sugar
MD	-	molecular dynamics
NMR	-	nuclear magnetic resonance
HPLC	-	high-performance liquid chromatography
TLC	-	thin-layer chromatography

LIST OF SYMBOLS

%	-	percent
℃	-	degree celcius
β	-	beta
α	-	alpha
Å	-	angstrom
°	-	degree
≥	-	more than or equal
=	-	equal
>	-	more than

CHAPTER 1

INTRODUCTION

1.1 Background of Study

According to the "green chemistry" evolution concept, efficient utilization of raw materials is applied on to minimize waste by avoiding the use of toxic and/or hazardous substances that can lead to health, safety and environmental issues (Sheldon, 2014). Therefore, to exploit these raw materials in a sustainable approach by an economical and friendly manner, then biorefinery comes into the picture (Arevalo-Gallegos *et al.*, 2017). A lignocellulose biorefinery plan manages to produce various value-added biochemical products like ethanol, ethylene, sorbitol, xylitol, furfural, etc (Iqbal, Kyazze and Keshavarz, 2013). Examples of lignocellulosic biomasses are industrial crops which have dominated 89.5% of agricultural land in Malaysia (Arshad, 2017). Besides palm oil, rubber, and cocoa, kenaf has emerged as one of the potential industrial crops since 2000 for production of fibres, polymer composite, and paper (Cheng, Haque Akanda and Nyam, 2016; Alkbir *et al.*, 2016; Ashori, 2006).

Lignocellulose is composed of three main polymers which are cellulose (40-60 % of the total dry weight), hemicellulose (20-40 %) and lignin (10-25 %). These polymers are the root cause of the lignocellulose recalcitrance according to the high crystallinity and polymerization degree of cellulose (Lee, Hamid and Zain, 2014), heterogeneity of hemicellulose (Gírio *et al.*, 2010) and highly aromatic contained lignin (Schoenherr, Ebrahimi and Czermak, 2018). Thus, the degradation of lignocellulosic biomass is very complex and requires synergistic action of

hemicellulases, cellulases and ligninolytic enzymes (Andlar *et al.*, 2018). The second most abundant polymer of hemicellulose has a random and amorphous structure comprises xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan (Isikgor and Becer, 2015; Baeyens *et al.*, 2015). Thus, complete degradation of hemicellulosic polysaccharides requires the cooperation of multiple hemicellulases including endo-xylanases, β-xylosidases, α-L-araninofuranosidases, α-D-glucuronidases, and acetyl-xylan-esterases to break down xylan into linear xylooligosaccharides (Shallom and Shoham, 2003; Beg *et al.*, 2001; Madadi, Tu and Abbas, 2017).

Xylanase as one of the hemicellulases are grouped into glycoside hydrolase (GH) 10 and 11 based on amino acid sequence homologies and hydrophobic cluster analysis of CAZy database (Henrissat, 1991). GH11 xylanase has a lower molecular weight compared to the GH 10 family and it folds into a domain composed of two β-sheets (A and B) packed parallel to each other and one α-helix. The protein structure is similar to a partially closed right hand consisted of 'thumb', 'palm' and 'fingers' regions (Havukainen *et al.*, 1996; Törrönen and Rouvinen, 1997) (Figure 1.1). These regions are involved in substrate binding and catalysis and even in certain xylanase, they get assisted by the secondary binding site located in N-terminal region (Ludwiczek *et al.*, 2007). Due to the ability to hydrolyse the β-D-(1,4) xylosidic linkages in xylan, GH 11 xylanases have a great commercial interest in potential industrial applications of biorefinery. However, the nature of "biomass recalcitrance' of the lignocellulose is challenging for efficient hydrolysis by xylanase that leads to the increase of the enzyme costs but low yields sugar produced (Himmel *et al.*, 2007; Visser *et al.*, 2015).

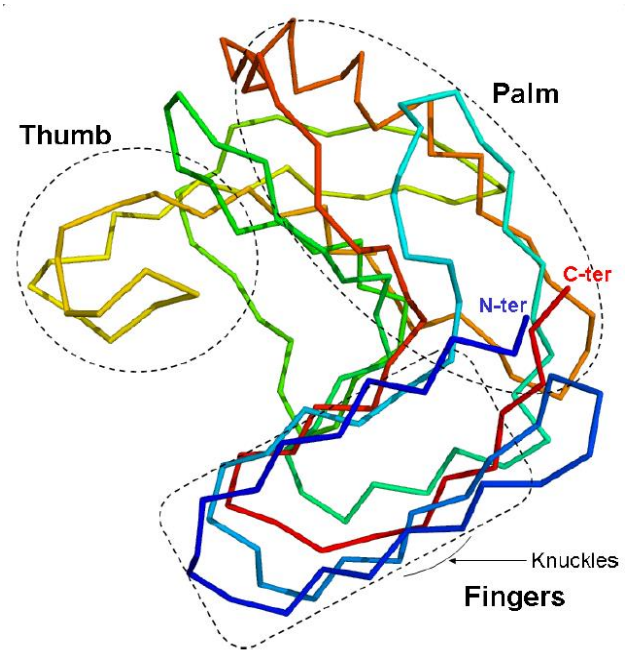

Figure 1.1 Ribbon representation of *Thermobacillus xylanilyticus* xylanase (Tx-Xyn) three-dimensional structure. The schematic protein is 'color-ramped' from the N-terminus (blue, N-ter) to the C-terminus (red, C-ter). The relevant regions of 'thumb', 'palm' and 'fingers' are highlighted in frames, and the 'knuckles' in the fingers region is indicated by an arrow (Song *et al.*, 2012).

To encounter the problem of inefficiency and low yields in enzymatic hydrolysis of lignocellulosic biomass, the catalytic efficiency of xylanase is needed to be increased first before performing cooperation action with cellulases (Morone and Pandey, 2014; Berlin *et al.*, 2005). As a solution, protein engineering can be applied on using one of the approaches that are rational design which is more suitable for the improvement of activity, stability or substrate specificity of the target enzyme. Plus, it encounters the problem of large clone libraries needed by random mutagenesis which causes a troublesome for high-throughput screening (Chica, Doucet and Pelletier, 2005; Martinez and Schwaneberg, 2013). However, researchers discovered that the most successful strategy is the combination of random and focused mutagenesis (Packer and Liu, 2015). For instances, the combination of N-terminal region replacement and site-directed mutagenesis at the cord of xylanase has significantly improved the specific activity (5.3-fold increase), substrate affinity and catalytic efficiency (Li *et al.*, 2017). Another study by Hoffmam *et al.*, 2016 showed that the

fusion of a carbohydrate-binding module from GH 6 resulted in 65 % increase of catalytic efficiency of GH11 xylanase and led to the 17 % increase of sugar release from pretreated sugarcane bagasse hydrolysis. Plus, error-prone PCR mutagenesis in combination with site saturation mutagenesis at H179 residue improved k_{cat}/K_m of xylanase to 3.46-fold (Wang *et al.*, 2013).

Previously, one of the potential GH11 xylanase isolated from *Aspergillus fumigatus* RT-1, afxynG1 (GenBank accession no: GQ458016) showed a great thermostability by retaining 70% of its activity after 30 minutes incubation at 70°C compared to its optimum temperature at 50°C (Abdul Wahab, Jonet and Illias, 2016). However, the sugar produced from the hydrolysis of the lignocellulosic substrate is very low. Thus, it is necessary to improve the catalytic efficiency of AfxynG1 for better hydrolytic performance. The main focus of this study is to improve the catalytic efficiency of AfxynG1 using protein engineering for producing a better yield of sugar. Error-prone PCR, site-saturation and site-directed mutagenesis approaches led to the amino acid substitutions of AfxynG1 within the N-terminal region and substrate binding site of the AfxynG1. The result showed that the mutations acquired increased catalytic efficiency (k_{cat}/K_M) and improved thermostability and acid stability. Therefore, the production of total reducing sugar was enhanced up to 28.6 % from pretreated kenaf hydrolysis. In consequences, these mutants have a great potential in lignocellulosic biomass saccharification for large industrial applications.

1.2 Problem Statement and Gap of the Study

The recalcitrance of lignocellulose structure becomes a drawback for enzymatic hydrolysis due to the complex and heterogeneous structure of cellulose, hemicellulose and, lignin. A previous study used two-step pretreatment process involves calcium hydroxide (Ca(OH)2), and peracetic acid (PPA) had successfully removing a major part of the lignin layer and maintaining most of the hemicellulose

of kenaf (Wan Azelee *et al.*, 2014). This hemicellulose part is ready to be degraded by hydrolytic enzymes for fermentable sugar production.

Multiple hydrolysis enzymes are required to function synergistically in saccharification of the lignocellulosic biomass for biorefinery industry. One of the main enzymes involved is xylanase which is still lack of study compared to the cellulases. The most studied xylanases concerning lignocellulosic degradation are involved in a mixture or synergism with other hydrolytic enzymes (Jia *et al.*, 2015; Yang *et al.*, 2015). Besides, the genetic modification for xylanase improvements are more focus on the high temperature and extreme pH tolerance (Li *et al.*, 2015; Boonyapakron *et al.*, 2017) but the catalytic enhancement is still very little. In the meantime, only one study was focus to improve the catalytic efficiency of xylanase towards natural substrate hydrolysis (Song *et al.*, 2012).

Thus, the main target of this study is to improve the catalytic efficiency of the xylanase to produce a higher yield of sugar from the lignocellulosic substrate hydrolysis. The knowledge regarding substrate binding and catalysis of the xylanase must be explored to enable the genetic modification of the suitable amino acid residues. Furthermore, the enhancement of thermostability and pH stability is required to prepare a high potential enzyme for industrial use.

1.3 Objectives of the Study

The objectives of this research are stated as below;

1) To identify substrate binding residues that involve and important for substrate binding and catalysis of AfxynG1

2) To improve the catalytic efficiency of AfxynG1 for hydrolysis of pretreated kenaf and biochemically characterise the AfxynG1 mutants

1.4 Scopes of the Study

This study focuses on the improvement of catalytic efficiency of a GH11 xylanase from *A. fumigatus* RT1 (AfxynG1) using directed evolution of epPCR, site saturation and site-directed mutagenesis. The epPCR acts as a fine tuner to select potential residues for SSM following two strategies: 1) residues that shared by more than one mutant and 2) residues which near to catalytic and substrate binding site. The second strategy needs structural information using molecular docking to identify the substrate-binding residues. All of the clones from epPCR and SSM libraries undergo high-throughput and low-throughput screening towards pretreated kenaf hydrolysis to isolate the potential mutants. Multiple mutants from three single improved mutants are constructed employing site-directed mutagenesis and screened towards pretreated kenaf. All of the final mutants are expressed and partially purified for biochemically characterised and kinetic determination. The reducing sugar produced from pretreated kenaf hydrolysis for each mutant was compared.

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APPENDICES

APPENDIX A

Medium, solutions and buffers preparation

Appendix A1: Medium Preparation

1.	Luria Bertani (LB) medium	100 ml
	Bacto-tryptone	1 g
	Bacto-yeast extracts	0.5 g
	NaCl	0.5 g

To make LB agar, the same ingredient used with addition 1.5 g agar before autoclaving.

2.	TSS Reagent	40 ml
	Bacto-tryptone	0.4 g
	Bacto-yeast extract	0.2 g
	NaCl	0.2 g
	Polyethylene glycol (PEG)	4.0 g
	Dinethyl sulfoxide	2.0 ml
	$MgCl_2$	0.194 g

Appendix A2: Autoinduction medium

1. Luria-Bertani for Autoinduction (LBA) medium

Composition: 1% w/v tryptone, 0.5% yeast extract, 0.1% NaCl

For 1 liter LBA medium

1) The following ingredients were dissolved in 950 ml distilled water: 10 g tryptone, 5 g yeast extract, 1 g NaCl .
2) Distilled water was added to bring the volume to 1 liter
3) The solution was autoclaved for 15 min at 121 °C
4) The solution has cooled to ~ 55 °C before used.

2. 20X NPS stock solution

Composition: 0.5 M $(NH_4)_2SO_4$, 1 M KH_2PO_4, 1 M Na_2HPO_4.

For 1 liter 20 X NPS stock solution:

1) The following ingredients in sequence in beaker; stir until all were dissolved in 900 ml distilled water:

Component	1 liter	Conc. at 1 x
dH_2O	900 ml	
$(NH_4)_2SO_4$	66	25 mM
KH_2PO_4	136	50 mM
Na_2HPO_4	142	50 mM

2) Distilled water was added to bring the volume to 1 liter
3) The solution was autoclaved for 15 min at 121 °C
4) The solution has to be cooled to ~ 55 °C before used.

3. 50 X 5052 Stock solution

Composition (5052= 0.5% glycerol, 0.05% glucose, 0.2% α-lactose)

For 1 liter 50 X 5052 stock solution:

1) The following ingredients in sequence in beaker; stir until all were dissolved in 900 ml distilled water:

Component	1 liter
Glycerol (weight in beaker)	250 g
H_2O	730 ml
Glucose	25 g
α-lactose	100 g

4. 1000X trace metals mixture stock solution

(100 ml in ~50 mM HCl)

1) All metal stock solutions were prepared in miliQ H_2O, except for $FeCl_3$, which is dissolved in ~0.1M HCl, as noted in the table below. Combine the metal solutions as in the table below:

Component	Volume	MW	1 X concentration
H_2O	36 ml	-	-
0.1 M $FeCl_3.6H_2O$ *yellow* (dissolved in ~0.1M HCl)	50 ml	270.30	50 µM Fe
1 M $CaCl_2$	2 ml	110.99	20 µM Ca
1M $MnCl_2.4H_2O$	1 ml	197.91	10 µM
1 M $ZnSO_4.7H_2O$	1 ml	287.56	10 µM Zn
0.2 M $CoCl_2.6H_2O$ *pink*	1 ml	237.95	2 µM Co
0.1 M $CuCl_2.2H_2O$ *blue*	2 ml	170.486	2 µM Cu
0.2 M $NiCl_2.6H_2O$ *green*	1 ml	237.72	2 µM Ni
0.1 M $Na_2MoO_4.2H_2O$	2 ml	241.98	2 µM Mo
0.1 M $Na_2SeO_3.5H_2O$	2 ml	263.03	2 µM se
0.1 M H_2BO_3	2 ml	61.83	2 µM H_3BO_3

2) The stock solutions of the individual metals were autoclaved, except for 0.1 M FeCl2 in 1/100 volume of concentrated HCl.
3) For Na_2SeO_3, a brief precipitate appeared upon addition, which redissolved rapidly.
4) The stock were stored at room temperature.

5. $MgSO_4$ stock solution

Composition: 1 M $MgSO_4$

For 100 ml of 1M $MgSO_4$ stock solution:

1) The $MgSO_4.7H_2O$ powder was weight to 24.65 g and dissolved in 100 ml distilled water.
2) The solution was filtered using a sterile 0.2 µm nylon syringe filter and keep in a sterile vial.
3) The solution was stored in room temperature prior to use.

6. LBA-5052 medium

1) Contains glucose to supress expression while growing to high density, lactose to induce expression when glucose and glycerol exausted. NB metals should be added before addition of NPS to avoid precipitation.
2) The following ingredients were dissolved in ~ 928 ml LBA:

Component	**1 liter**
LBA	~ 928 ml
1 M $MgSO_4$	1 ml
1000 X metals (use 0.1 x)	100 µl
50 x 5052	20 ml
20 x NPS	50 ml
Ampicillin (100 mg/ml)	1 ml

Appendix A3: Antibiotic and solutions for enzyme expression

1. Ampicillin stock solution

Composition: 100 mg/ml ampicillin**.**

For 10 ml ampicillin stock solution:

1) The ampicillin powder was weight to 1 g and dissolved in 10 ml distilled water.
2) The solution was filtered using a sterile 0.2 µm nylon syringe filter and keep in a sterile vial.
3) The solution was stored in -20 °C prior to use.

2. IPTG stock solution

Composition: 0.5 M IPTG

For 10 ml IPTG solution:

1) The IPTG powder was weight to 1.19 g and dissolved in 100 ml distilled water.
2) The solution was filtered using a sterile 0.2 µm nylon syringe filter and keep in a sterile vial.

The solution was stored in -20 °C and thawed prior to use

Appendix A4: Solutions for Molecular Works

1. 50X TAE Electrophoresis Buffer — 2 L

   (2 M Tris, 50 mM EDTA)

Tris base	484 g
Glacial acetic acid	114.2 ml
0.5 M EDTA, pH 8.0	200 ml

   To make 1X TAE 20 L, add 400 ml 50X Buffer into 19.6 L of distilled water.

2. 10X TE Buffer — 100 ml

Tris base	100 mM
EDTA, pH 8.0	10 mM

   To make 1X TE Buffer, dilute 1 ml of 10X TE Buffer with 9 ml of distilled water.

3. Tris-Cl, pH 8.0 — 500 ml

Tris	60.57 g

   Add 350 ml distilled water and adjust pH to 8.0 with concentrated HCl (approximately 21 ml, but start with less). Let it cool to room temperature and make final adjustment to the pH. Autoclavable.

4. 0.5 M EDTA, pH 8.0 — 500 ml

EDTA	93.05 g

   Dissolve in 350 ml distilled water. Place on a magnetic stirrer and stir vigorously. Adjust the pH to 8.0 by adding approximately 10 g NaOH pellets. The disodium salt of EDTA will not go into solution until the solution is adjusted to approximately pH 8.0. Bring to 500 ml total volume with distilled water. Filter and sterilize by autoclaving.

Appendix A5: Working Solutions for SDS-PAGE

1.	Acrylamide Mix Stock Solution 30% (w/v) Acrylamide 0.8% (w/v) Bis-acrylamide	100 ml
2.	10% SDS	100 ml
	Sodium dodecyl sulfate	10 g
	Distilled water	100 ml
	Store at room temperature.	
3.	10% Ammonium persulfate	5 ml
	Ammonium persulfate	0.5 g
	Distilled water	5 ml
	Stable for months in a capped tube in refrigerator.	
4.	10% Separating Gel	5 ml
	Distilled water	1.9 ml
	30% Acrylamide mix	1.7 ml
	1.5 M Tris-HCl, pH 8.8	1.3 ml
	10% SDS	0.05 ml
	10% Ammonium persulfate	0.05 ml
	TEMED	0.002 ml

5.	5% Stacking Gel	5 ml
	Distilled water	3.4 ml
	30% Acrylamide mix	0.83 ml
	1 M Tris-HCl, pH 6.8	0.63 ml
	10% SDS	0.05 ml
	10% Ammonium persulfate	0.05 ml
	TEMED	0.005 ml

6.	Tris-glycine Electrophoresis Buffer	1 L
	Tris	3 g
	Glycine	14.4 g
	SDS	1 g

Use distilled water to make 1 liter solution. pH should be approximately 8.3.

7.	5X Sample Buffer	10 ml
	1 M Tris-HCl, pH 6.8	0.6 ml
	50% Glycerol	5 ml
	10% SDS	2 ml
	2-mercaptoethanol	0.5 ml
	1% Bromophenol blue	1 ml
	Distilled water	0.9 ml

Stable for weeks in the refrigerator or for months at -20ºC.

8.	Staining Solution (Solution A)	1 L
	Methanol	450 ml
	Distilled water	450 ml
	Glacial acetic acid	100 ml
	Coomassie Blue R-250	1 g

9.	Destaining Solution (Solution B)	1 L
	Distilled water	800 ml
	Methanol	100 ml
	Glacial acetic acid	100 ml

Appendix A6: Working Solutions for Western Blot

1. Blocking Solution	20 ml
5% skimmed milk	1 g
Add with 1X TBST until 20 ml	

2. 10X TBS	1 L
Tris-base	24.2 g
NaCl	80 g
Adjust to pH 7.5	

3. 1X TBSTT (Ab 1:2000)	
1X TBS	200 ml
Tween-20	100 µl

Appendix A7: pH Buffers

1. Glycine-HCl (pH 3)

0.2 M glycine	12.5 ml
0.2 M HCl	2.85 ml

Top up to 50 ml with ddH2O.

2. Sodium acetate (pH 4)

0.2 M acetic acid	164 ml
0.2 M sodium acetate	36 ml

Top up to 200 ml with ddH2O.

3. Sodium acetate (pH 5)

0.2 M acetic acid	59 ml
0.2 M sodium acetate	141 ml

Top up to 200 ml with ddH2O

4. Sorensen phosphate (pH 6)

0.2 M NaH_2PO_4	44 ml
0.2 M Na_2HPO_4	6.2 ml

Top up to 50 ml with ddH2O

5. Sorensen phosphate (pH 7)

0.2 M NaH_2PO_4	39 ml
0.2 M Na_2HPO_4	61 ml

Top up to 100 ml with ddH2O

6. Sorensen phosphate (pH 8)

0.2 M NaH_2PO_4	5.3 ml
0.2 M Na_2HPO_4	94.7 ml

Top up to 100 ml with ddH2O

7. Glycine -NaOH (pH 9)

0.2 M NaOH	4.4 ml
0.2 M glycine	25 ml

Top up to 50 ml with ddH2O

Appendix A8: DNS Assay Buffer

Dinitrosalicylic acid (DNS) solution	1 L
3,5-dinitrosalicylic acid	10 g
Sodium potassium tartrate tetrahydrate	300 g
2N NaOH	200 ml

Add 3,5-dinitrosalicylic to 500 ml of reagent grade water. Add slowly sodium potassium tartrate tetrahydrate and 2 N NaOH. Dilute to a final volume of 1 L with reagent grade water. Protect from carbon dioxide and store no longer than 2 weeks.

Appendix A9: Protein Purification buffer

1. Buffer A

Composition: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 30 mM imidazole

2. Buffer B

Composition: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 300 mM imidazole

3. Stripping buffer

Composition: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 50 mM EDTA

4. Phenylmethylsulfonyl fluoride (PMSF)

Composition: 10 mM PMSF.

For 10 ml 10 mM PMSF:

1) The PMSF powder was weight to 17.4 mg and dissolved in 10 ml isopropanol
2) The solution was filtered using a strile 0.2 µm nylon syringe filter and keep in a sterile vial.
3) The solution was aliquots of appropriate volume and stored in -20 °C prior to use.

Note:The half-life of a 20 µM aqueous solution of PMSF is about 35 minutes at pH 8.

APPENDIX B

XYLOSE STANDARD

Plotting graph for xylose standard

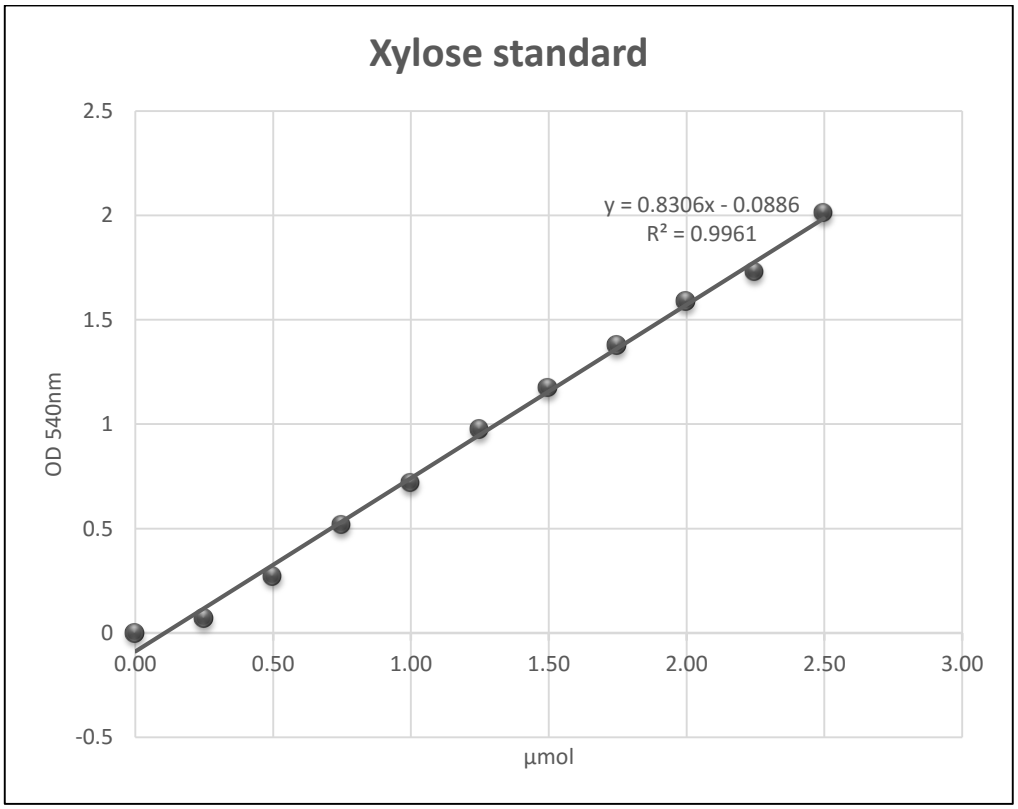
1) Prepare 1 mg/ml xylose stock in 50 mM sodium acetate buffer (pH 5). Prepare different concentrations of xylose as stated below:

Concentration (mg/ml)	Volume of xylose(µl)	Volume of buffer(µl)
0	0	750
0.05	37.5	712.5
0.1	75	675
0.15	112.5	637.5
0.2	150	600
0.25	187.5	562.5
0.3	225	525
0.35	262.5	487.5
0.4	300	450
0.45	337.5	412.5
0.5	375	375

2) Run the standard xylose as DNS assay of xylanase in triplicate.
3) Record the readings of OD at 540 nm and convert the xylose concentration to µmol as stated below:

Xylose concentration (mg/ml)	Xylose in µmol	Readings of at OD 540
0.00	0.00	0.00
0.05	0.25	0.07
0.10	0.50	0.27
0.15	0.75	0.52
0.20	1.00	0.72
0.25	1.25	0.98
0.30	1.50	1.17
0.35	1.75	1.38
0.40	2.00	1.59
0.45	2.25	1.73
0.50	2.50	2.01

4) Plotting the graph of xylose (µmol) vs OD at 540 nm.



5) Use the equation from the graph for calculation of xylanase enzyme activity as 1U of enzyme is defined as the amount of enzyme releasing 1 µmol xylose per min.

APPENDIX C

BSA STANDARD

Plotting graph for xylose standard

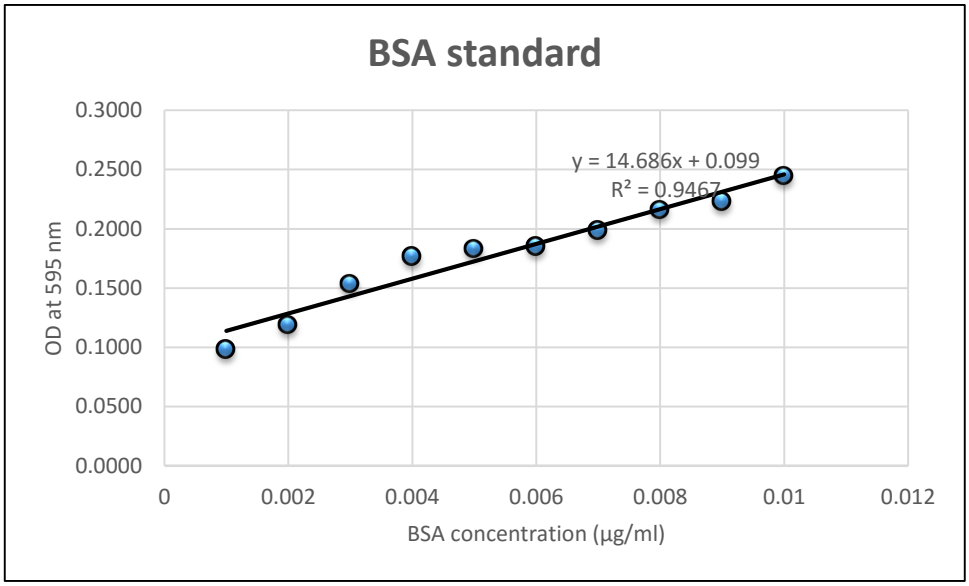
1) Prepare 2.5 µg/ml BSA stock in 50 mM sodium acetate buffer (pH 5.0) and prepare a set concentration of BSA as stated below:

BSA concentration (µg/ml)	Volume of BSA (µl)	Volume of buffer (µl)
0.001	0.2	99.8
0.002	0.4	99.6
0.003	0.6	99.4
0.004	0.8	99.2
0.005	1.0	99.0
0.006	1.2	98.8
0.007	1.4	98.6
0.008	1.6	98.4
0.009	1.8	98.2
0.01	2.0	98.0

2) 100 ml BSA at varied concentration is mixed with 100 ml Bradford reagent at RT in the 96-flat bottom plate. The mixture is equally mixed by pipetting. The triplicate mixture is prepared for each of BSA concentration.
3) The colorimetric readings are measured by multiwell plate reader immediately at 595 nm absorbance.
4) Record the readings as stated in the table below:

BSA concentration (µg/ml)	OD readings at 595 nm
0.001	0.0983
0.002	0.1190
0.003	0.1537
0.004	0.1770
0.005	0.1830
0.006	0.1850
0.007	0.1985
0.008	0.2160
0.009	0.2230
0.01	0.2447

5) Plotting the graph of BSA concentration (µg/ml) vs OD at 595 nm.



6) Use the equation from the graph for calculation of concentration of xylanase

APPENDIX D

KINETIC PARAMETER MICHAELIS-MENTEN

The derivation of the Michaelis–Menten equation assumes that a slow, product forming reaction follows the rapid, reversible formation of an enzyme–substrate complex:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \overset{k_2}{\rightarrow} \mathrm{E} + \mathrm{P}, \qquad \textbf{Equation E11}$$

Where, E is the enzyme, S is the substrate and P is the product. The Michaelis–Menten equation is then derived by using the steady-state approximation for the ES complex: specifically the concentration of the enzyme–substrate complex is assumed to change much more slowly than the concentration of the substrate, so the rate equation takes the form.

$$\frac{d[\mathrm{P}]}{dt} = \frac{V_{\max}[\mathrm{S}]}{K_{\mathrm{m}} + [\mathrm{S}]} \qquad \textbf{Equation E12}$$

Where, K_m is the Michaelis constant and V_{max} is the maximum velocity of the reaction achieved when the enzyme active sites in the sample are all complexed with substrate all the time, and [P] is the concentration of product at any given time during the time course. The relationship between the K_m and the unitary rate constants in the reaction scheme is:

$$K_{\mathrm{m}} = \frac{k_{-1} + k_2}{k_1} \qquad \textbf{Equation E13}$$

Taking the reciprocal of both sides of the Michaelis–Menten equation gives the Lineweaver–Burk equation that is often used to graphically analyse enzyme kinetic data. The equation is:

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \qquad \textbf{Equation E14}$$

This relationship was used to estimate V_{max} and K_m values.

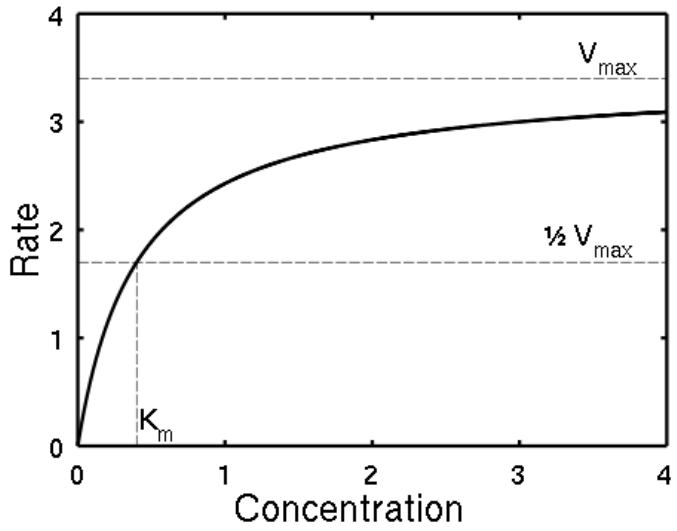


Figure D1: Plot of rate of reaction versus the substrate concentration.

The Michaelis-Menten constant (K_m) and maximum velocity of substrate hydrolysis (V_{max}) were determined from the Lineweaver-Burk plot

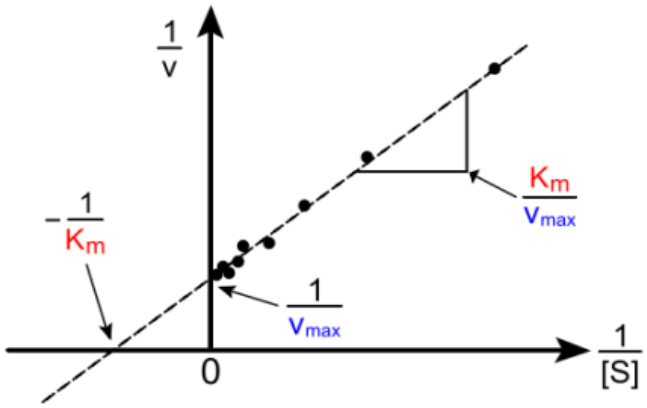


Figure D2: The Lineweaver-Burk plot and kinetic parameters determination.

LIST OF PUBLICATIONS AND PAPER PRESENTED

1. **Damis, S.I.R**., Abd. Murad, A.M., Abu Bakar, F.D., Rashid, S.A., Jaafar, N.R., and Illias, R.M. (2019). Protein engineering of GH11 xylanase from *Aspergillus fumigatus* RT-1 for catalytic efficiency improvement on kenaf biomass hydrolysis. *Enzyme and Microbial Technology*. 131 [109383].

2. **Damis, S.I.R**, Illias, R.M. (2016). Substrate-binding site recognition of family 11 xylanase from *Aspergillus fumigatus* by molecular docking. *International Conference on Bioinformatics and Computational Biology*. Feb 2-3, 2016. Kuala Lumpur.

3. Noor, Y.M., Samsulrizal, N.H., Jema'on, N.A., Low, K.O., Ramli, A.N., Alias, N.I., **Damis, S.I.R**, Fuzi, S.F., Isa, M.N., Murad, A.M., Raih, M.F., Bakar, F.D., Najimudin, N., Mahadi, N.M., and Illias RM. (2014). A comparative genomic analysis of the alkalitolerant soil bacterium *Bacillus lehensis* G1. *Gene*. 545(2):253-61.